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Diploid Male Production Results in Queen Death in the Stingless Bee *Scaptotrigona depilis*

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Abstract As in most Hymenoptera, the eusocial stingless bees (Meliponini) have a complementary sex determination (CSD) system. When a queen makes a "matched mating" with a male that shares a CSD allele with her, half of their diploid offspring are diploid males rather than females. Matched mating imposes a cost, since diploid male production reduces the colony workforce. Hence, adaptations preventing the occurrence or attenuating its effects are likely to arise. Here we provide clear evidence that in the stingless bee *Scaptotrigona depilis*, the emergence of diploid males induces queen death, and this usually occurs within 10–20 days of the emergence of diploid male offspring from their pupae. Queens that have not made a matched mating die when introduced into a colony in which diploid males are emerging. This shows that the adult diploid males, and not the queen that has made a matched mating herself, are the proximate cause of queen death. Analysis of the cuticular hydrocarbon profiles of adult haploid and diploid males shows six compounds with significant differences. Moreover, the diploid and haploid males only acquire distinct cuticular hydrocarbon profiles 10 days after emergence. Our data shows that the timing of queen death occurs when the cuticular hydrocarbons of haploid and diploid males differ significantly, suggesting that these chemical differences could be used as cues or signals to trigger queen death.

Keywords Sex determination · Diploid male load · Queen execution · Chemical recognition

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Introduction

Male Hymenoptera are normally haploid and typically develop from non-fertilized eggs, while females are diploid and develop from fertilized eggs. Nevertheless, in many species, diploid individuals can develop into males as a result of the complementary sex determination (CSD) system that is characteristic of many Hymenoptera. Under this system, individuals that are heterozygous at the CSD locus develop as females, whereas haploid (hemizygous) individuals or individuals that are homozygous at the CSD sex locus develop into males (Whiting 1943). Since diploid males are usually nonviable or sterile (Heimpel and de Boer 2008), females that make a matched mating and mate with a male sharing one of her two sex alleles will end up having reduced fitness. In small populations, the increased frequency of matched mating due to reduced allelic diversity at the CSD locus may also increase extinction risk (Zayed and Packer 2005). In eusocial species,

this effect is further exacerbated by the fact that males do not participate in the colony tasks and that the replacement of workers with diploid males will therefore reduce colony performance (Heimpel and de Boer 2008).

Mechanisms preventing the production of diploid males are relatively common amongst Hymenoptera species, and are mainly related to inbreeding avoidance, i.e., reducing the chances of making a matched mating (van Wilgenburg et al. 2006). Some eusocial species also have mechanisms to reduce the cost of matched matings and diploid male production (van Wilgenburg et al. 2006). In the honeybee, *Apis mellifera* for example, there are two of such mechanisms. First, because honeybee queens are highly polyandrous, the variance in diploid male production among queens is reduced. Although this results in a higher proportion of queens making a matched mating, it reduces the proportion of diploid male offspring derived from each matched mated queen (Page and Metcalf 1982). Second, honeybee workers detect diploid male larvae and eliminate them within 36 h of hatching (Woyke 1962, 1963). This avoids wasting resources in rearing them and allows the cell to be quickly reused (Ratnieks 1990). By contrast, stingless bees (Meliponini) rear brood in sealed cells that are mass provisioned with food and have no option to detect and eliminate diploid male larvae in an early stage (Camargo 1979). However, it has been suggested that stingless bees evolved an alternative mechanism to reduce the costs of diploid male load, namely by executing queens that have made a matched mating. Queen execution by the workers is strongly suspected in *Melipona quadrifasciata* and *M. scutellaris*, where young matched-mated queens died 25–30 and 11–67 days, respectively, after their diploid male offspring first began to emerge (Alves et al. 2011; Camargo 1976).

Matricide is a rare behaviour in a social insect species, since replacing a queen with her daughter causes a reduction in relatedness, and hence a loss in inclusive fitness, both to the executed queen and to the executing workers (Ratnieks et al. 2006). This behaviour, however, occurs in some social species with annual life cycles near the end of the season (Bourke 1994), when worker-worker relatedness is high and so favors worker production of males (Loope 2015). In the case of queen execution in stingless bees due to diploid male production, it is likely that both queens and workers benefit from queen replacement. That is because the 50% reduction in worker production might be very detrimental to colony reproduction, and since swarms in eusocial bees are highly dependent on colony population size (Rangel and Seeley 2012), they may lead to low fitness levels. Alves et al. (2011) suggested that queen execution could reduce the negative fitness effects of diploid male production, as it would usually result in the matched-mated queen being replaced by a daughter who was not matched mated. However, further investigations are necessary to verify whether the execution of a matched-mated queen leads to a successful queen replacement under natural conditions, and consequently an increase in the inclusive fitness of the executed queen. Matched matings in stingless bees can occur whenever a young queen mates, both during queen supersedure in established colonies and during colony fission via swarming. When killing a matched-mated queen increases the inclusive fitness of the executing workers we could expect them to use cues or signals either from the queen or from diploid males to trigger execution.

Quantitative differences in the chemical profiles of diploid and haploid first instar male larvae have been demonstrated to provide the information allowing workers to detect diploid males in the honey bee (Santomauro et al. 2004). Likewise, a potential source of information for triggering queen death in stingless bees is any difference in the chemical profile of haploid versus diploid males. No such difference has been observed between newly emerged diploid and haploid *M. quadrifasciata* males (Borges et al. 2012). However, newly emerged individuals usually do not have a fully mature cuticle and have lower amounts of cuticular hydrocarbons than older individuals (Falcón et al. 2014; LeConte and Hefetz 2008), which could explain the lack of differences observed in their chemical profiles. In addition, there are two alternative potential sources of information that might trigger queen death: (i) the matched mated queen could release a signal herself resulting from mating with a male with the same sex allele; or (ii) the presence of too many males emerging at the same time, independent of their ploidy.

In this study, we investigated whether the emergence of diploid male offspring triggers the death of the mother queen in *Scaptotrigona depilis* and if it is the matched mated queen herself or the presence of diploid males that triggers this behaviour. In addition, we analyzed the cuticular chemical profiles of haploid and diploid males as a function of age and tested if the time at which differences arise coincides with the timing of queen execution. We also estimated the proportion of queens that made matched matings and from this estimated the effective number of alleles at the CSD locus in our study population.

Methods and Materials

Study Species and Site The study was carried out using colonies of *Scaptotrigona depilis* kept in hives at the experimental meliponary of the Biology Department at the University of São Paulo, Ribeirão Preto, Brazil. *Scaptotrigona depilis* is a naturally occurring species there and it is common on the USP RP campus. A recent census over nine years (http://www.apacame.org.br/mensagemdoce/100/artigo3.htm, accessed on 16/01/2017) found 109 colonies nesting in hollow trees in the 5.8 km^2 campus. In addition, a further 80–90 colonies were kept in hives. Colonies are perennial, containing thousands of workers and are headed by a single-mated queen (Paxton et al.

2003). Data collection and analyses were carried out from September 2012 to November 2014.

Obtaining Matched Mated Queens In order to obtain queens that had made a matched mating, we first removed the laying queen from 68 colonies of *S. depilis*. This resulted in a new queen taking over and performing a mating flight. Approximately 30 days after the new queen started laying eggs, her oldest offspring are now pupae with black eyes. At this time, we inspected samples of pupae taken from brood cells in order to identify colonies in which approximately 50% of the pupae had the distinctive male morphology of smaller heads and bigger eyes (Fig. SM1).

Five queens producing c. 50% male offspring were obtained and were marked by clipping their wings (Fig. SM2). In these colonies, ten males were collected and checked using DNA microsatellite genotyping at three loci to confirm that they were diploid (see below). We prevented the execution of queens that had made a matched mating by repeatedly removing them from their own colonies and placing them in colonies without diploid males, from which we had removed the queen, so that the colonies they were in never had diploid males.

Time Course of Queen Replacement In order to determine the time course of natural queen replacement we studied 43 of the 68 replacement queens resulting from the process (see above) of obtaining matched mated queens. We inspected the hives every 10 days after removing the original queen in order to determine whether a new physogastric (i.e., egg laying) queen was present or not.

Effective Number of Sex Alleles in the Population The effective number of sex alleles n present in our study population was estimated based on the estimator $n = 2/m$, where m is the observed proportion of matched matings (Cook and Crozier 1995). The 95% confidence intervals were estimated based on the exact expected confidence intervals of a binomial proportion (Clopper and Pearson 1934).

Queen Execution Bioassays To determine whether a queen dies after the emergence of diploid males from brood cells, we removed the matched mated queen from her colony a few days before diploid male emergence was due to start and replaced her with a non-matched mated marked queen (see Fig. SM3 and SM4 for more details). We then inspected the colony every ten days for 50 days. We determined that a queen had died by observing that the colony was queenless, when either there was no production of new brood cells or by observing the presence of a new, non-marked, physogastric queen.

Our matched-mated queens were kept alive through multiple cycles of brood rearing by introducing each queen into a new queenless colony every 35 days, the average time taken for egg to adult development. As a result, we were able to repeat the queen execution bioassays four times for each of the five matched mated queens, to give 20 repetitions. The survival of the queens that had made a matched mating and which were placed in colonies not producing diploid males over a period of 35 days could be used to conclude if signals produced by the queens themselves did or did not induce queen death. As a control for the use of non-nestmate introduced queens, we also removed queens that had not made a matched mating and replaced these with another queen that had not made a matched mating. We carried out 20 repetitions, this time using 20 different removed queens and 20 different replaced queens.

It was not necessary to record data blind with respect to the colony type (i.e., with or without diploid males) because our study involved a binary observation (queen died or did not die) and so it is not subject to any error or bias in categorization.

Chemical and Genetic Analyses Combs from three different colonies classified as producing or not producing diploid males based on previous genetic analysis were collected and placed in petri dishes in an incubator at 28 °C until the adult males emerged. Subsequently, adult males were placed in petri dishes in groups of eight, fed with sugar syrup and pollen ad libitum, and kept until five or ten days of age.

We analyzed 38 diploid males, from three different matched mated queens, 12 aged zero days, 12 with five days and 14 with ten days. Two of these were later shown by genetic analyses (see below) to be haploid and were removed from our analysis. The 41 haploid males analyzed were from ten different queens that were not producing diploid male offspring, n = 12, 12 and 17 in the same three age groups. The males were then freeze killed at −20 °C and then individually extracted in 1.0 ml of pentane for ten minutes. The solution was evaporated at room temperature (23 to 27 °C) and resuspended in 150 μl pentane. Samples were analyzed on a Shimadzu QP 2010 Ultra couple gas chromatograph/mass spectrometer with a DB-5 ms capillary column (30 m × 0.25 mm × 0.25 μm film thickness) by injecting 2 μl of our extract. We used an initial column temperature of 70 °C held for one minute, increasing to 150 °C at a rate of 20 °C min^{-1}, and then to 320 °C at 3 °C min^{-1}. The final temperature of 320 °C was held for 15 min with a final pressure of 75 kPa. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. Samples were run using splitless injection and an inlet temperature of 280 °C. The electron ionization voltage was auto-tuned to enhance the acquisition performance according to the molecular weight of the compounds, and the ion source temperature was set to 300 °C. Peaks in the chromatogram were integrated using GCMS Solutions software, and compounds were identified based on their retention times and expected diagnostic ions in the mass spectra as well as through comparison with known standards and library searches, using the NIST 11 mass

spectral database. Double bond positions of alkenes were identified through dimethyl disulfide (DMDS) derivatization. Relative peak areas were calculated based on the total ion chromatogram after the exclusion of contaminants and background noise from the chromatograms.

After cuticular hydrocarbon extraction, individuals were kept in absolute ethanol for genetic analysis to confirm their ploidy. We genotyped all males from diploid male producing colonies, because matched mated queens may also lay haploid eggs that develop into haploid males (Paxton et al. 2003). Some, but not all haploid males were genotyped to assure the reliability on the analysis, since non-matched mated queens produce only haploid males. DNA was extracted using the Chelex method and males were genotyped at five microsatellite loci, T1, T4, T8 (Paxton et al. 1999), and Sxant06 and Sxant18 (Duarte et al. 2011). Microsatellite amplification and visualization were done as described previously by Francisco et al. (2011). We categorized males as diploid if they were heterozygous at one or more loci (Alves et al. 2011).

Statistical Analyses All statistical analyses were performed using R 3.2.2 (R core team 2015). The survival probability of matched mated queens versus unmatched mated queens was assessed using generalized linear models (GLM) with a binomial error distribution in which treatment group (presence or absence of emerging diploid males) and day of observation (0, 10, 20, 30, 40 and 50) were included in a full factorial heterogeneity of slopes model. Wald Z tests were used to assess significance.

For the chemical analyses, the integrated raw peak area of the chemical compounds was transformed according to Aitchison (1982) using the centered log-ratio transformation to decrease the dependence that is otherwise inherent in compositional data. In order to investigate whether particular cuticular hydrocarbons could be used as a cue for workers to discriminate between haploid and diploid males we performed linear models in which the abundance of a given chemical compound was the dependent variable, age and ploidy groups independent variables, and colony a random factor. These models were fit using R package *lme4* 1.1.7, after which posthoc tests were performed using function *glht* in the *multicomp* package (ver. 1.4–0) and *p*-values were *FDR* corrected for multiple comparisons using the Benjamini & Hochberg method using the *p.adjust* function. In addition, the overall multivariate differences in chemical profiles among age and ploidy groups were determined using Permanova with 1000 permutations (package *vegan* 2.3–0).

Results

Time Course of Queen Replacement Following removal of the egg-laying queen from 43 colonies, 35 (81%) had a new physogastric queen when the colonies were inspected for the first time ten days later, and the other eight at the 20 day inspection.

Effective Number of Sex Alleles in the Population We observed five matched matings out of 68 matings (7.35%). Taking into account that, as in most other stingless bees, queens in *S. depilis* are nearly always single mated (Paxton et al. 2003), we estimated that there are therefore 27.2 sex alleles in the population (exact 95% binomial confidence interval: [12.2–82.3]).

Queen Execution Bioassays All 20 queens (none of whom had themselves made a matched mating) that were transferred into colonies with emerging diploid males, died, presumably by execution. Three (3/20, $n = 15\%$) had died by the ten day inspection, 14 (70%) by the 20 day inspection, and the last three (15%) by the 30 day inspection (Fig. 1). In all cases, queen death was followed by queen replacement. By contrast, only one out of 20 control replacement queens died, within ten days of replacement (Fig. 1). A binomial GLM confirms that the survival probability of queens in a colony with emerging diploid males was therefore significantly lower than of queens in a colony without emerging diploid males (*binomial GLM*, $Z = -4.124$, $P < 0.001$). In the 20 trials where a queen that had made a matched mating was transferred to a colony not producing diploid males, they survived for at least 35 days. These results clearly demonstrate that the information that triggered the mother queens' death was not caused by a signal produced by the queens themselves, but instead was due to the production of the higher number of adult diploid males. We should note that we were unable to observe queen execution directly. However, given the evidence for virgin queen execution by workers in many stingless bees species (Imperatriz-Fonseca and Zucchi 1995; Kärcher et al. 2013; Wenseleers et al. 2004), the most probable reason for queen death was execution by the workers. However, further investigations are necessary to determine exactly how the mother queens died.

Genetic Analyses Genetic analyses of male offspring from the five queens producing large numbers of male offspring confirmed that they had made matched matings as their male progeny were diploid. Two males used for the chemical analysis were found to be haploid (Table S1) and were therefore excluded from our chemical analyses.

Chemical Analyses Thirty-one hydrocarbon peaks were identified on the cuticles of *S. depilis* males, and included both saturated and unsaturated alkanes and alkenes (Table S2). The cuticular chemical profiles of diploid and haploid only show differences 10 days after of emergence, but not at 0 or 5 days, in which six compound peaks show significant differences in their relative abundance between haploid and diploid males

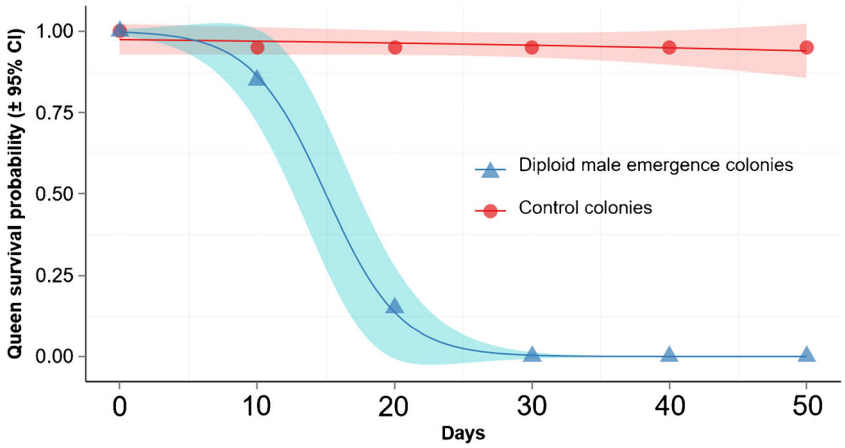

Fig. 1 Survival of *Scaptotrigona depilis* queens that replaced matched-mated queens where excess diploid males were produced ($n = 20$) versus that of non-matched mated queens placed in colonies where no diploid males were produced (control) ($n = 20$). The probability of queen survival with the 95% confidence intervals (shaded areas) per group are indicated at each time point

(Fig. 2; Fig. SM5). Some were relatively more abundant on haploid males: a mixture of branched heptacosanes (13-, 11-, 9-, 7- MeC_{27}) and two nonacosenes (11-$C_{29:1}$ and 5-$C_{29:1}$). Others were more abundant on the cuticle of diploid males: 9-pentacosene ($C_{25:1}$), 7-octacosene ($C_{28:1}$), and 3,15-dimethylheptacosane (3,15-diMeC27). Both age and ploidy significantly affected the overall cuticular chemical profile (age: *Permanova*, F = 10.25, $P < 0.0001$; ploidy: *Permanova*, F = 3.85, $P = 0.003$; age and ploidy interaction: *Permanova*, F = 3.44, $P < 0.0001$). Aging affects the cuticular chemical composition somewhat similarly for haploid and diploid males with the differences between the two groups

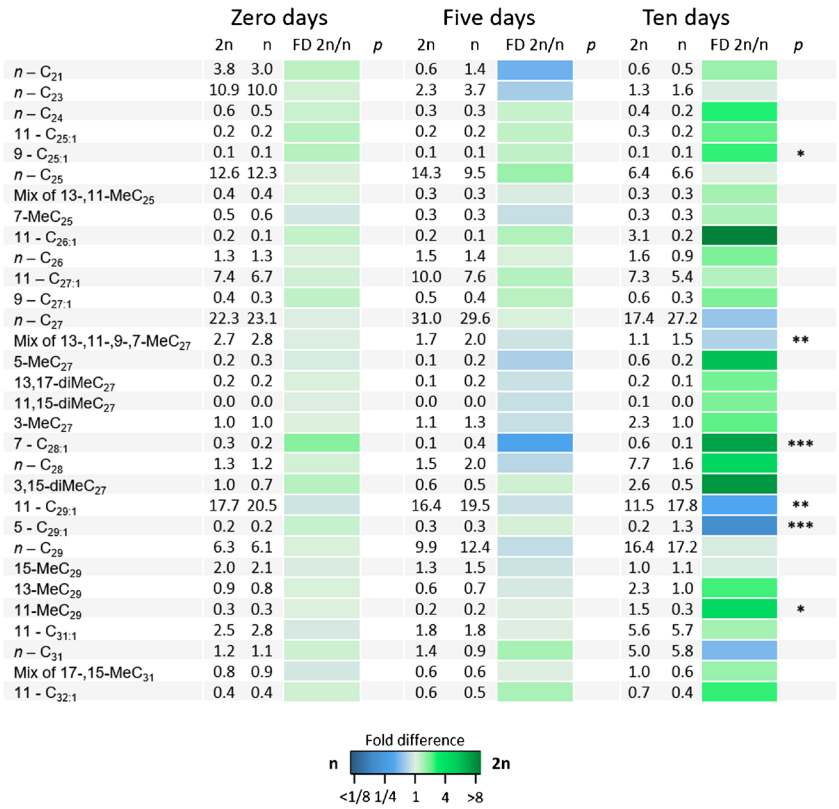

Fig. 2 Percent relative abundance of cuticular hydrocarbons present in *Scaptotrigona depilis* haploid (n) and diploid (2n) males of three different age classes (newly emerged, five and ten days old). The heatmap shows the fold difference in the average relative abundance of the different compounds in diploid vs. in haploid males for males belonging to different age classes. Chemical compounds with colors towards the blue pallet were overrepresented in haploid males, whereas substances with colors towards the green pallet were overrepresented in diploid males. No significant differences were observed between newly emerged haploid and diploid males. *P* values indicate *FDR* corrected significance levels: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. A complete table with chemical compound areas together with effect sizes and significance levels is presented in supplementary Table S2

being more pronounced only at the age of 10 days, indicating that only when the cuticle is fully mature workers would be able to assess male ploidy (Fig. 2; Fig. SM5). Therefore, our data suggest that chemical information could be used by workers to detect the occurrence of diploid males in their colony (Fig. 2, Table S2).

Discussion

Our data show that *S. depilis* queens transferred into a colony producing adult diploid males die, presumably executed by the workers, within 10 to 30 days of the start of diploid male emergence. This is very similar to the situation in two *Melipona* species (Alves et al. 2011; Camargo 1976), and suggests that the death of queens that have made a matched mating at the CSD locus is not confined to *Melipona* in the stingless bees. Since the queens that died did not actually make a matched mating but were transferred into colonies producing diploid males, our results also show that the information triggering queen death is not present on the matched-mated queen but arises from the diploid males themselves. Additionally, queens that had made a matched mating that were transferred to colonies not producing diploid males all survived. Our study is the first to systematically examine queen death in relation to matched matings and diploid male production, and suggests that this may well be a general phenomenon among many more, if not all species of stingless bee (Alves et al. 2011; Camargo 1976).

The cuticular hydrocarbons of diploid and haploid males were significantly different in males only 10 days after emergence from their sealed cells and not immediately after emergence or after 5 days. This suggests that the cue, or signal, that triggers queen death is the presence in the colony of males with a distinct diploid-male odour. Alternatively, it is possible that the workers might execute the queen due to the presence of an unusually large numbers of males in the colony. In fact, it is expected that workers would replace the queen if high numbers of males are produced, as in other species where this is frequently used as a cue for queen sperm depletion (Baer et al. 2016; Butler 1957). However, because queen execution mostly took place from 10 to 20 days after the start of the emergence of diploid males, this would be too soon for the number of males in the colony to build up to their maximum level (i.e., the haploid males +50% of the diploid adults), unless the workers could simply assess that there were “too many young males” rather than “too many males in total”. Moreover, queen execution in response to the mere overproduction of males is incompatible with the fact that in *S. depilis* colonies, as in many other stingless bees, males are produced in distinct cohorts, resulting in male production reaching ca. of 30% during periods of pronounced male production (Bego 1982). This would make male overproduction an unreliable signal of whether or not a mother queen had made a matched mating, as regular haploid male production would then frequently induce queen death.

Diploid males are chemically distinct from diploid female workers, both qualitatively and quantitatively. Female workers present a more complex CHC profile in terms of the diversity of chemical classes of compounds, which includes alcohols, ketones and esters, while only hydrocarbons occur on both haploid and diploid males (Oliveira et al. 2015; Poiani et al. 2014). This indicates that diploid males could indeed have a noticeable odour inside the colony, different from female workers and haploid males. The time when queens started to die coincided with the timeframe during which the diploid males started to acquire a specific cuticular profile, further indicating that male chemical profile differences are involved in queen death. The few cases in which queens took longer than 20 days to die could be explained by the fact that the number of diploid males inside the colony still needed to reach a certain threshold before triggering the queen’s death, or even that diploid males’ chemical profile kept changing as they aged for longer than 10 days. Earlier evidence that diploid males are recognized based on their chemical profile comes from the honey bees *A. mellifera* and *A. cerana,* where workers are able to detect and eliminate diploid male within a day or two after hatching (Santomauro et al. 2004; Woyke 1962, 1963, 1979;). We are unsure though why, in our study system, evolution would not result in a more efficient system being selected for, where, for example, diploid males would acquire specific chemical profiles at a much earlier age. One possibility is that intrinsic physiological constraints limit the evolutionary possibilities, as also in many other insects, cuticular profiles only mature after a certain period of time (Falcón et al. 2014; van Zweden and d'Ettore 2010). Further research is needed to determine whether adult diploid males signal their diploid status in *S. depilis*. What is clear, however, is that the relative amounts of several compounds present on the cuticle of diploid males differed with that present on haploid males. Although no qualitative differences were found, workers of social insects are definitely able to detect quantitative differences in cuticular chemical profiles, in contexts such as nestmate recognition, egg policing, and queen pheromones (e.g. Oi et al. 2015a, 2015b; Smith et al. 2009; van; Van Oystaeyen et al. 2014; van Zweden and d'Ettore 2010). In the honeybee, the cannibalism of diploid male larvae is likely triggered by an odd pattern of five different chemical compounds, mostly alkenes, present on the cuticle (tricosane, pentacosene, heptacosene, nonacosene and squalene) (Santomauro et al. 2004). In our study, some alkenes were also differently expressed on the cuticle of diploid males including some of the same compounds found in the honeybees, namely pentacosene and nonacosene. It is possible that this is due to conservation of the underlying biosynthetic pathways in honeybees and stingless bees, although study of a larger set

of species and quantification of conservation in the underlying biosynthetic desaturases would be required to formally demonstrate this.

Our results suggest that queens of *S. depilis* are not able to discriminate against males carrying the same sex alleles prior to mating, at least at some degree, since ca. of 7% of the mating flights were matched in the studied population. Matched matings can arise due to mating with kin, with 50% of brother-sister matings resulting in a matched mating. Inbreeding was unlikely to contribute to the likelihood of making matched matings, since male stingless bees are known to mate near over long population-wide distances (Cameron et al. 2004; dos Santos et al. 2016). The fact that we worked with a managed population in an apiary kept at fairly high densities could in principle have influenced the probability that queens would have made a matched mating, though this is unlikely because our study species occurs naturally in high densities in hollow trees at the university campus (http://www.apacame.org.br/mensagemdoce/100/artigo3.htm, accessed on 16/01/2017). In addition, our estimate on the proportion of matched matings is not significantly different from earlier estimates by Paxton et al. (2003), with the same species (known as *S. postica* at that time) and location, in which two out of ten colonies were producing diploid males, giving an estimate of ten sex alleles (Fischer's exact test of mating data, $P = 0.2188$). Our results also showed no significant differences with estimates from other studies of stingless bees: four matched mated queens out of 49 matings in *M. compressipes fasciculata* (Kerr 1987; Fischer's exact test, $P = 1$); one out of five in *Tetragonula carbonaria* (Green and Oldroyd 2002; Fischer's exact test, $P = 0.4091$); two out of 38 in *M. scutellaris* (Alves et al. 2011; Fischer's exact test, $P = 1$); three out of 28 in *M. seminigra merrilae* (Francini et al. 2012; Fischer's exact test, $P = 0.7027$); and two out of 30 in *M. interrupta manaosensis* (Francini et al. 2012; Fischer's exact test, $P = 1$).

Diploid male load, due to *complementary sex determination*, is a crucial selective pressure acting in both social and solitary Hymenoptera. Meliponini bees have monandrous queens which results in 50% of their diploid brood being male if they make a matched mating. This high proportion will almost certainly greatly reduce colony performance especially if the males are not detected and killed in the early larval stage, as in honey bees. Our results greatly strengthen the hypothesis that queen stingless bees are executed in response to making a matched mating, leading to queen replacement and the survival of the colony. Future studies would be required to fully measure the fitness consequences of queen elimination in terms of colony survival and performance, and pinpoint in detail which of the chemical compounds that differ between haploid and diploid males are actually used to recognize them and infer that a matched-mated queen is present in the colony.

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